AD_____

Award Number: DAMD17-01-1-0510

TITLE: Study of RANKL Expression in Metastatic Breast Carcinoma

PRINCIPAL INVESTIGATOR: Pardeep Bhatia, Ph.D.

CONTRACTING ORGANIZATION: University of Connecticut Health Center
Farmington, Connecticut 06030

REPORT DATE: June 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Annual (1 Jun 01 - 31 May 02)

4. TITLE AND SUBTITLE
Study of RANKL Expression in Metastatic Breast Carcinoma

5. FUNDING NUMBERS
DAMD17-01-1-0510

6. AUTHOR(S)
Pardeep Bhatia, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Connecticut Health Center
Farmington, Connecticut 06030

E-Mail: bhatiapardeep@hotmail.com

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Bone is the most common site of metastases by human breast cancer. Most breast cancers form osteolytic metastases, in contrast to tumors such as prostate cancer that form osteosclerotic metastases. Although some evidence suggests that formation of bone metastases by breast cancer cells is mediated by the increased osteoclastogenesis at the target site, a clear controversy exists whether formation of bone metastases is mediated by breast cancer cells directly or by stimulated osteoclasts. We have therefore examined the expression of RANK and RANKL, two proteins importin the bone remodeling signaling pathway, in invasive carcinoma of breast and bone metastases of breast. We observed both RANK and RANKL were upregulated in these breast tumors and metastases. Further, breast tumor cells were directly in contact with the bone without any osteoclasts in the vicinity. We suggest that overexpression of RANK and RANKL in breast cancer cells provides a growth advantage to the breast tumor cells, and the tumor cells appear to be directly responsible for the degradation of bone.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast carcinoma, metastasis, RANKL	13
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Bone is one of the most common target sites for cancer metastasis. Tumors such as breast carcinoma have a predilection for metastasizing to bone to form osteolytic lesions. Two models have been postulated to explain the bone destruction associated with breast cancer metastasis: the osteolysis is mediated either directly by tumor cells, or indirectly by osteoclasts. Cancer cells have been demonstrated to induce osteoclast differentiation and proliferation by several osteotrophic factors such as IL-1, IL-6, LIF, prostaglandin E2, tumor necrosis factor-α and parathyroid hormone related protein (PTHrP) or by direct cell-cell interaction with bone marrow cells (Mundy 1997, Guise, 1997, Guise and Mundy 1998, Thomas et, 1999). Recently, Receptor Activator of NF-kB ligand (RANKL) and its receptor RANK, have been identified and implicated in regulation of bone remodeling (Holfbauer et al., 2001). RANKL is expressed on stromal cells and osteoblasts and is thought to mediate the interaction between these cells and pre-osteoclasts by binding to its receptor RANK which is expressed on the pre-osteoclasts. This interaction leads to osteoclastogenesis and bone resorption (Holfbauer et al., 2001). Thus the RANK and RANKL interaction appears to play a key role in the process of osteoclastogenesis and bone resorption. Expression of RANKL in other tumor types such as prostate cancer has also been reported (Brown et al., 1999). In addition, studies in mouse model system has generated evidence for the involvement of RANKL in metastatic bone destruction. (Guise 1997, Guise and Mundy 1998). Therefore my hypothesis was that in breast carcinoma expression of RANKL might correlate with bone metastasis and osteolysis.

Specific Aims:

- To examine different histological forms of breast carcinoma as well as metastatic breast cell lines for expression of RANKL and correlate this expression with phenotype.
- If RANKL expression is detected, to test the ability of anti-RANKL antibodies to inhibit the formation of osteolytic lesions in a model system

Body

It is well established that breast cancers have the capability to establish and grow as metastases in bone, however, the mechanism underlying their ability to induce osteolysis remains uncertain. *In vitro* studies have demonstrated that breast cancer cells alone have the capacity to degrade the bone matrix, although these lesions of bone or dentine slices are not of the magnitude of those resulting from osteoclast-mediated bone destruction (Eilon and Mundy, 1978). Studies have also shown that PTHrP is expressed by the metastatic breast cancer cells and is a critical component in the mechanism of breast cancer metastases to bone (Boyce et al., 1999; Chirgwin and Guise, 2000; de la Mata et al., 1995; Guise, 1997; Guise and Mundy, 1996; Guise et al., 1996; Guise et al., 1993; Henderson et al., 2001; Kohno et al., 1994; Thomas et al., 1999; Uy et al., 1995; Uy et al., 1997; Yoshida et al., 2000). Co-culture experiments have shown that breast cancer cells can produce both PTHrP and M-CSF which induce RANKL mRNA levels and inhibit OPG mRNA levels in osteoblasts *in vitro* (Mancino et al., 2001; Thomas et al., 1999).

We first investigated the expression of RANKL and its receptor RANK on an array of 60 primary and 10 bone metastatic breast tumors by immunohistochemistry using anti-RANKL and anti-RANK antibodies. These arrays of infiltrating ductal carcinoma (IDC) were obtained from Imgenex, (San Diego, CA) while bone metastatic tumors were obtained from the department of Pathology, University of Connecticut Health Center. Samples were prepared from paraffin embedded archival samples. They were cut using microtome and spread on polylysine coated slides. Tumors were stained using the standard protocol (Herrington and McGee,1992). Briefly slides were deparaffinized with xylene, dehydrated in alcohol and treated with 4N HCl at 37 C for 10 min. to retrieve the antigen. Samples were washed in distilled water and stained with anti-RANKL and anti-RANK antibodies using Histostain-SP kit (Zymed, South San Francisco, CA). Samples were mounted and photographed under the microscope. Same batch of tumors was also stained with H&E. We encountered a major problem in the antigen retrieving step. However, we have now been able to detect staining of both RANKL and RANK antigens.We observed that the breast cancer tumor cells directly invade the bone in the osteolytic lesions and no osteoclasts are present in the zone of lysis. (Fig1) We also observed that both primary Infiltrating Ductal carcinoma (IDC) as well as metastatic tumors overexpress RANKL and RANK in comparison to non-neoplastic breast (Fig 1-5).

Since we observed that normal breast epithelial cells as well as in primary and metastatic breast tumors express RANK on their cell surface, it is possible that during the formation of bony metastases, the metastatic tumor cells would express PTHrP and M-CSF which would stimulate adjacent osteoblasts to express RANKL and suppress OPG expression. This increased expression of RANKL on the surface of the osteoblasts, could interact with the RANK expressed on the surface of the breast cancer cells and stimulate the breast tumor cells to initiate osteolysis (Hunt et al., 2001). This destruction of the bone would release a variety of cellular growth factors such as TGF-β (Chirgwin and Guise, 2000; Yin et al., 1999) that could stimulate further growth by the breast cancer cells, leading to increased osteolysis and thus a stimulatory growth loop. Thus while it is likely that breast cancer tumor cells can act to stimulate osteoblasts to recruit osteoclasts

to an osteolytic lesion (Figure 6, Model 1), it is also possible that the breast cancer cells can themselves generate osteolytic lesions in the absence of osteoclasts by a direct interaction between the osteoblasts and the tumor cells (Figure 6, Model 2). In order to test this hypothesis, we are planning to study the expression of RANKL and RANK on human breast cancer cell lines. If we detect this expression, we intend to continue with our specific aim 2 and investigate whether breast tumor cells are also capable of in vitro bone resorption. If it does occur, we would like to further investigate whether anti-RANK or anti-RANKL antibodies could inhibit bone resorption by tumor cells.

KEY RESEARCH ACCOMPLISHMENTS:

- I found that breast cancer cells may directly invade the bone resulting in osteolytic lesions of bone metastasis
- I demonstrate that Receptor Activator of NF-kB ligand (RANKL) and its receptor RANK are expressed in primary as well as in bone metastatic tumors of breast carcinoma.
- I discovered that both RANK and RANKL are overexpressed in breast cancer cells of bony metastasis.
- This research supports a model by which formation of osteolytic lesions of bone metastasis by breast tumor cells may be due to the direct interaction of tumor cells which overexpress RANK with the stromal cells which express RANKL.

REPORTABLE OUTCOMES:

The data reported here was submitted as an abstract and presented as a poster at the Third North American Symposium on Skeletal Complications of Malignancy which took place on April 25-27, 2002 at NIH, Bethesda, Maryland. This abstract will be published in the journal Cancer Research supplement

CONCLUSION:

Breast cancer cells have the capability to establish and grow as metastasis in bone, however, the mechanism underlying their osteolysis is not understood. A controversy exists whether tumor cells are capable of osteolysis by themeselves or is this mechanism mediated by osteoclasts. In the present investigation we have observed that breast cancer cells in bone metastasis overexpress both RANKL and RANK. It is therefore possible that the interaction between RANK on the tumor cells and RANKL on the adjacent osteoblasts/stromal cells could lead to the formation of osteolytic lesions in bone. This may be an alternative mechanism to osteoclastic mediated bone resorption. The implication of this study is that, overexpressed RANK could be a drug target to inhibit bone metastasis by breast tumor cells. It is recommended that further studies are conducted in the animal model systems of breast cancer metastasis using anti-RANK antibodies to inhibit bone metastasis.

REFERENCES

Boyce, B. F., Yoneda, T., and Guise, T. A, *Endocr Relat Cancer* **6**, 333 (1999)

Brown J. M., Quinn J. E.,Buhler K. R., Vessella R. L, *J Bone Miner Res* **14**(Suppl 1), 1085 (2000)

Chirgwin, J. M., and Guise, T. A, *Crit Rev Eukaryot Gene Expr* **10**, 159 (2000)

Coleman, R. E., and Rubens, R. D, *Br J Cancer* **55**, 61 (1987)

De la Mata, J., Uy, H. L., Guise, T. A., Story, B., Boyce, B. F., Mundy, G. R., and Roodman, G. D, *J Clin Invest* **95**, 2846 (1995)

Eilon, G., and Mundy, G. R, *Nature* **276**, 726 (1978)

Guise, T. A, *Cancer* **80**, 1572 (1997)

Guise, T. A., and Mundy, G. R, *Curr Opin Nephrol Hypertens* **5**, 307 (1996)

Guise, T. A., Yin, J. J., Taylor, S. D., Kumagai, Y., Dallas, M., Boyce, B. F., Yoneda, T., and Mundy, G. R, *J Clin Invest* **98**, 1544 (1996)

Guise, T. A., Yoneda, T., Yates, A. J., and Mundy, *G. R., J Clin Endocrinol Metab* **77,** 40 (1993)

Henderson, M., Danks, J., Moseley, J., Slavin, J., Harris, T., McKinlay, M., Hopper, J., and Martin, T, *J Natl Cancer Inst* **93,** 234 (2001)

Herrington C.S. and McGee, J.O' D., *Diagnostic Molecular Pathology* (IRL, New York pp 8-17, 1992)

Holfbauer, L C., Neubauer, A ., and Heufelder, A.E, *Cancer* **92**,460 (2001)

Hunt, N. C., Fujikawa, Y., Sabokbar, A., Itonaga, I., Harris, A., and Athanasou, N. A, *Br J Cancer* **85**, 78 (2001)

Kohno, N., Kitazawa, S., Sakoda, Y., Kanbara, Y., Furuya, Y., Ohashi, O., and Kitazawa, R, *Breast Cancer* **1,** 43 (1994).

Mancino, A. T., Klimberg, V. S., Yamamoto, M., Manolagas, S. C., and Abe, E., *J Surg Res* **100**, 18 (2001)

Thomas, R. J., Guise, T. A., Yin, J. J., Elliott, J., Horwood, N. J., Martin, T. J., and Gillespie, M. T, *Endocrinology* **140**, 4451 (1999)

Uy, H. L., Guise, T. A., De La Mata, J., Taylor, S. D., Story, B. M., Dallas, M. R., Boyce, B. F., Mundy, G. R., and Roodman, G. D, *Endocrinology* **136,** 3207 (1995)

Uy, H. L., Mundy, G. R., Boyce, B. F., Story, B. M., Dunstan, C. R., Yin, J. J., Roodman, G. D., and Guise, T. A, *Cancer Res* **57**, 3194 (1997)

Yin, J. J., Selander, K., Chirgwin, J. M., Dallas, M., Grubbs, B. G., Wieser, R., Massague, J., Mundy, G. R., and Guise, T. A, *J Clin Invest* **103,** 197 (1999)

Yoshida, A., Nakamura, Y., Shimizu, A., Harada, M., Kameda, Y., Nagano, A., Inaba, M., and Asaga, T, *Breast Cancer* **7**, 215 (2000)

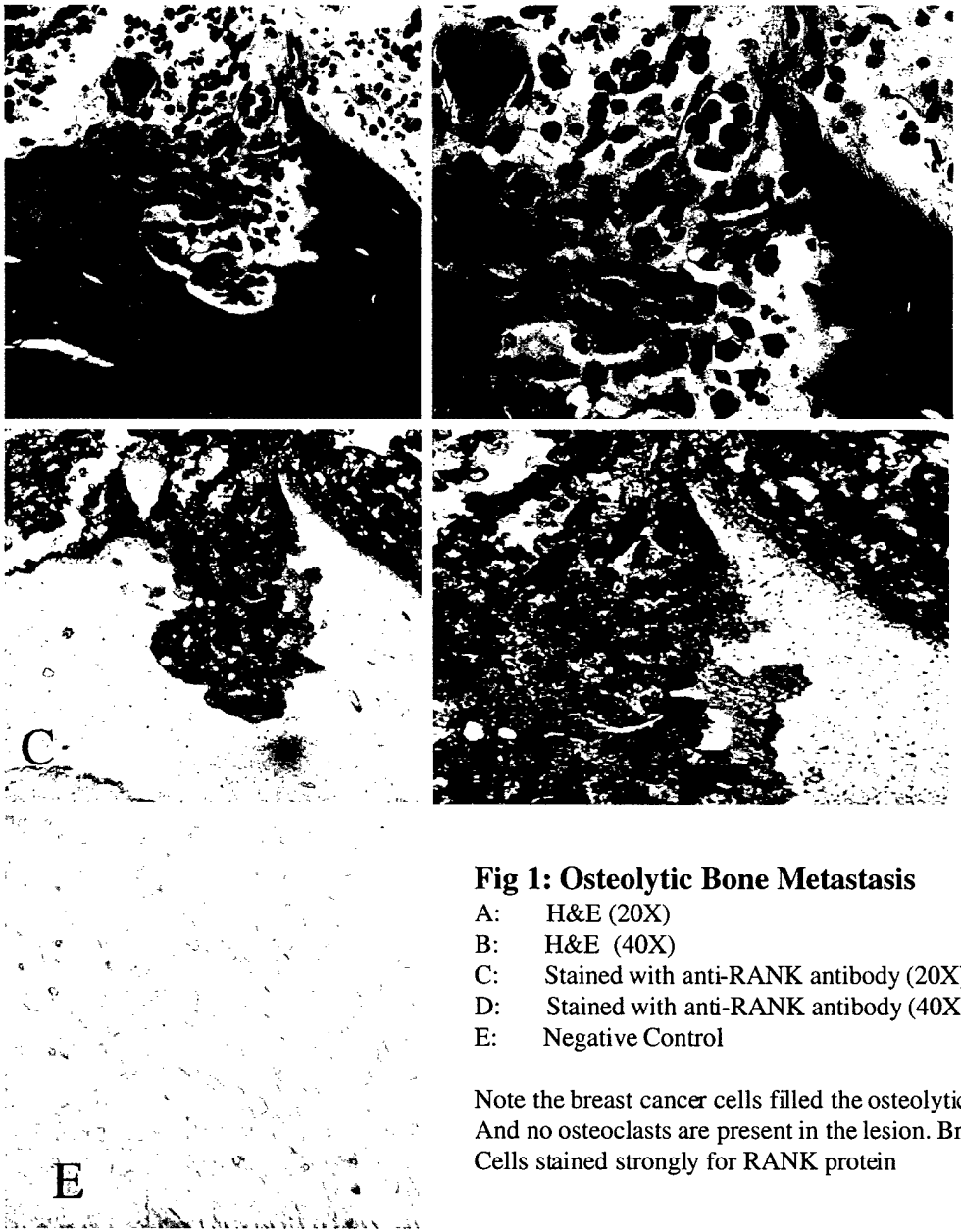


Fig 1: Osteolytic Bone Metastasis

A: H&E (20X)
B: H&E (40X)
C: Stained with anti-RANK antibody (20X)
D: Stained with anti-RANK antibody (40X)
E: Negative Control

Note the breast cancer cells filled the osteolytic lesions And no osteoclasts are present in the lesion. Breast cancer Cells stained strongly for RANK protein

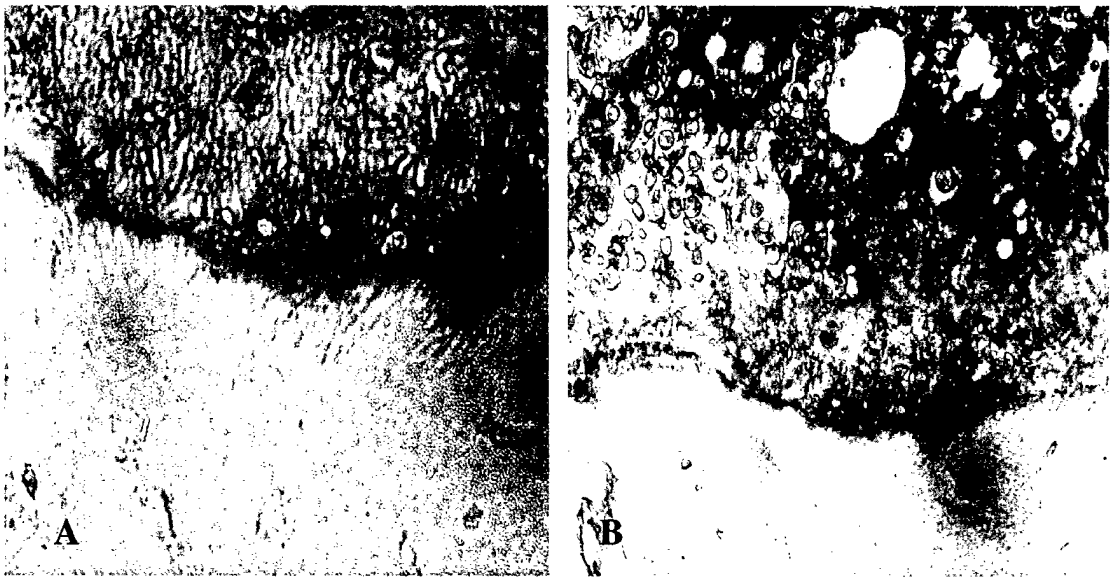


Fig 2: Osteolytic bone metastasis
A: RANKL (20X)
B RANKL (40X)

Osteoblasts cells stain strongly for RANKL
While the tumor cells although still positive
For RANKL stain less

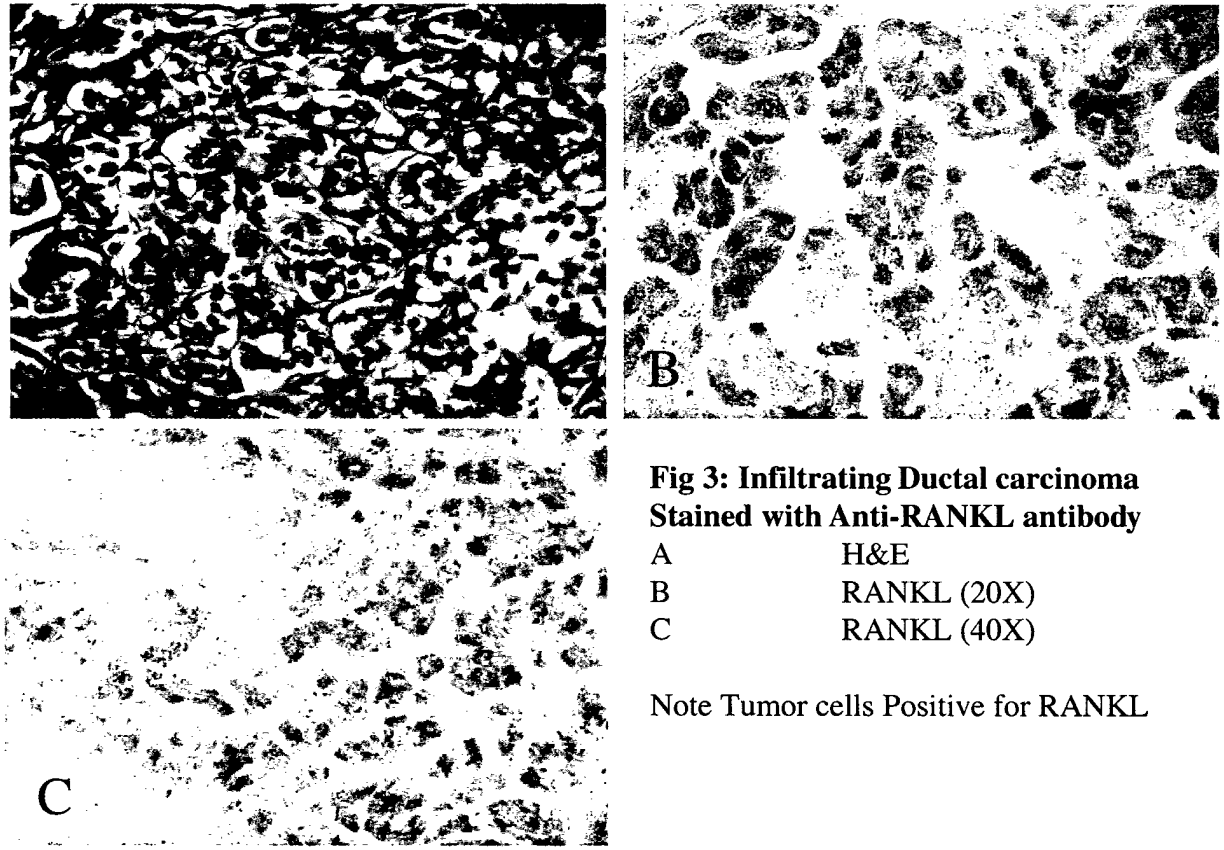


Fig 3: Infiltrating Ductal carcinoma Stained with Anti-RANKL antibody

A	H&E
B	RANKL (20X)
C	RANKL (40X)

Note Tumor cells Positive for RANKL

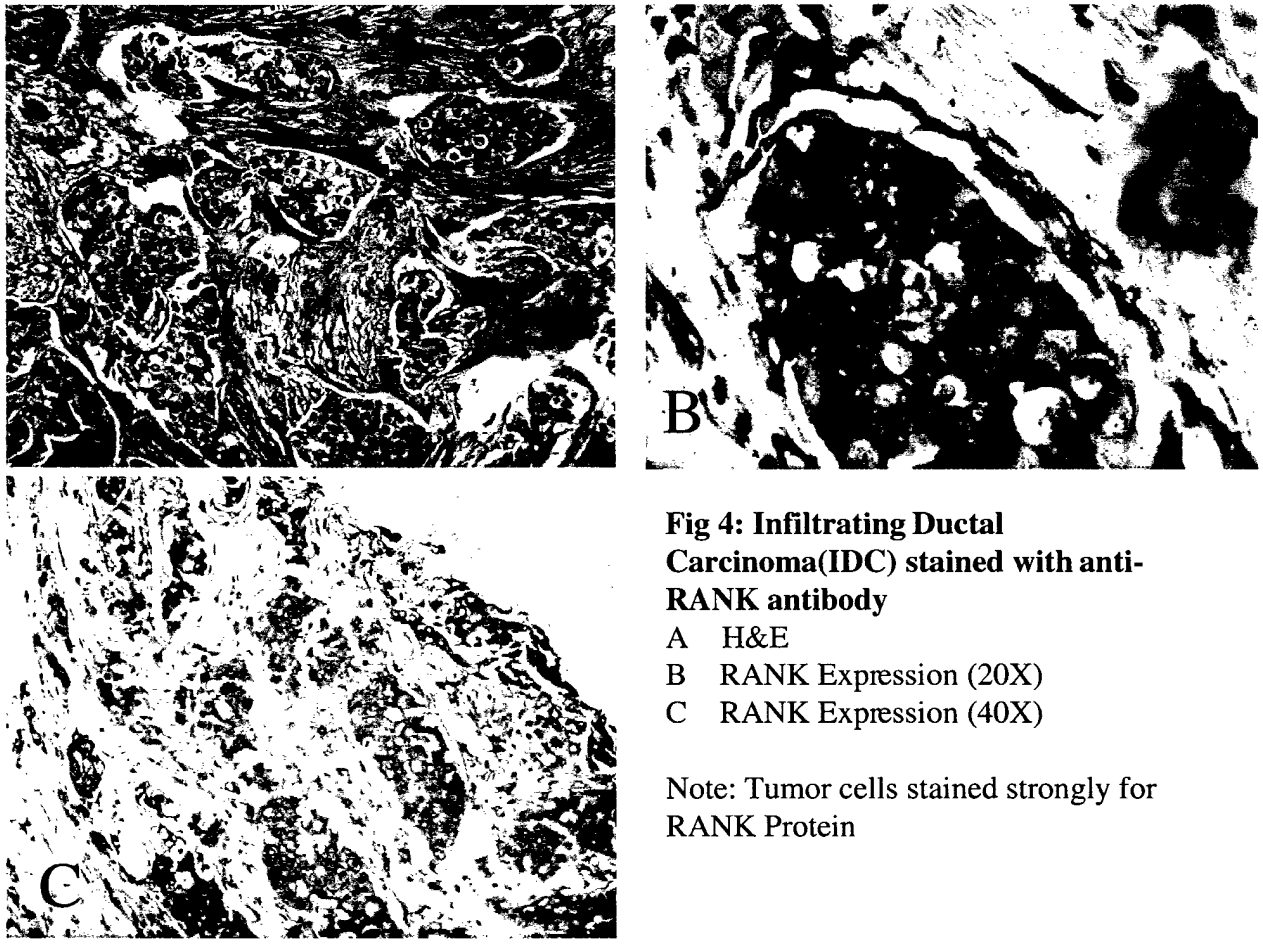
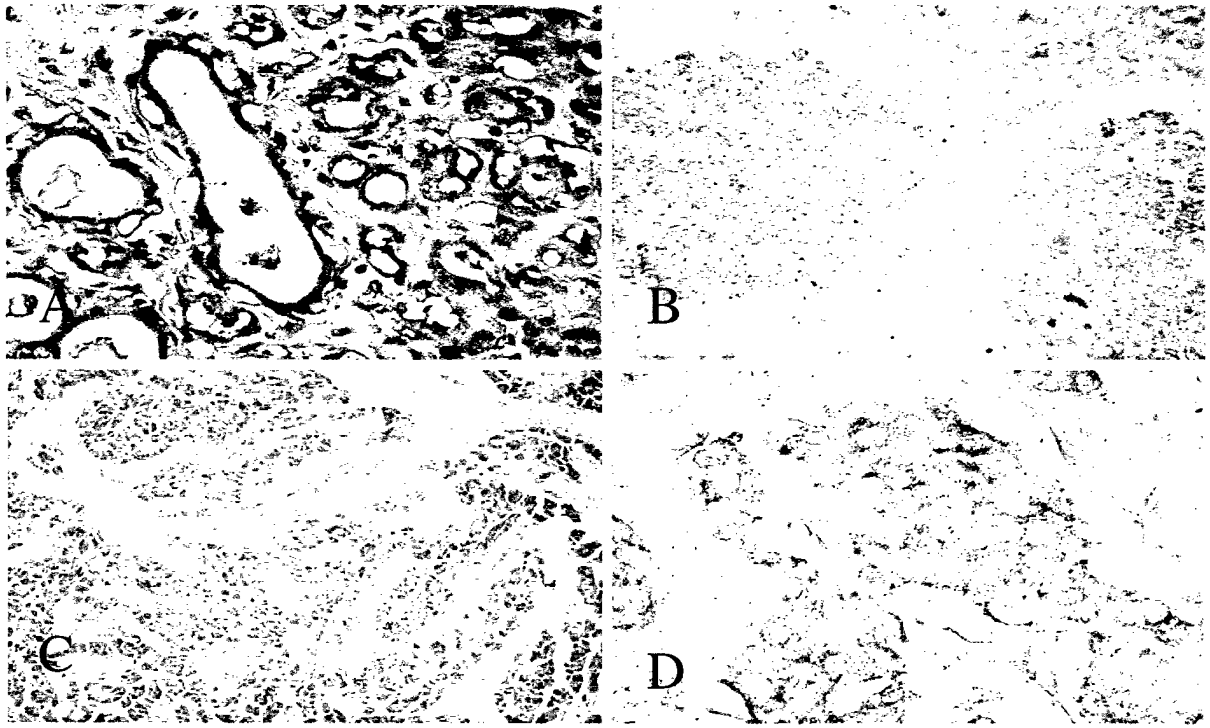


Fig 4: Infiltrating Ductal Carcinoma(IDC) stained with anti-RANK antibody

A H&E
B RANK Expression (20X)
C RANK Expression (40X)

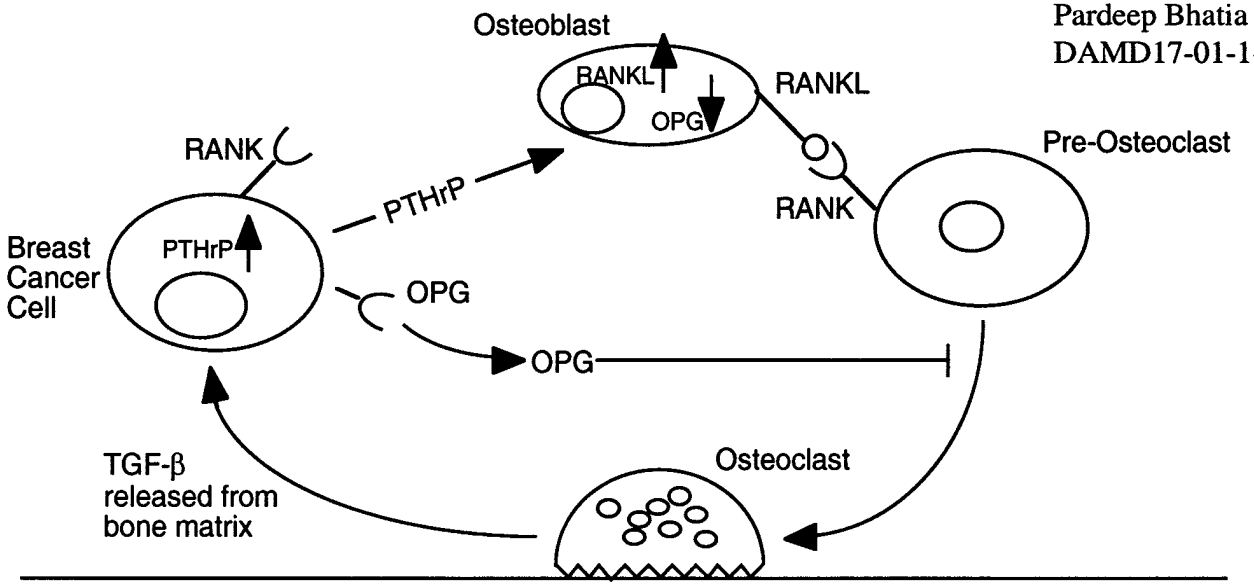
Note: Tumor cells stained strongly for RANK Protein



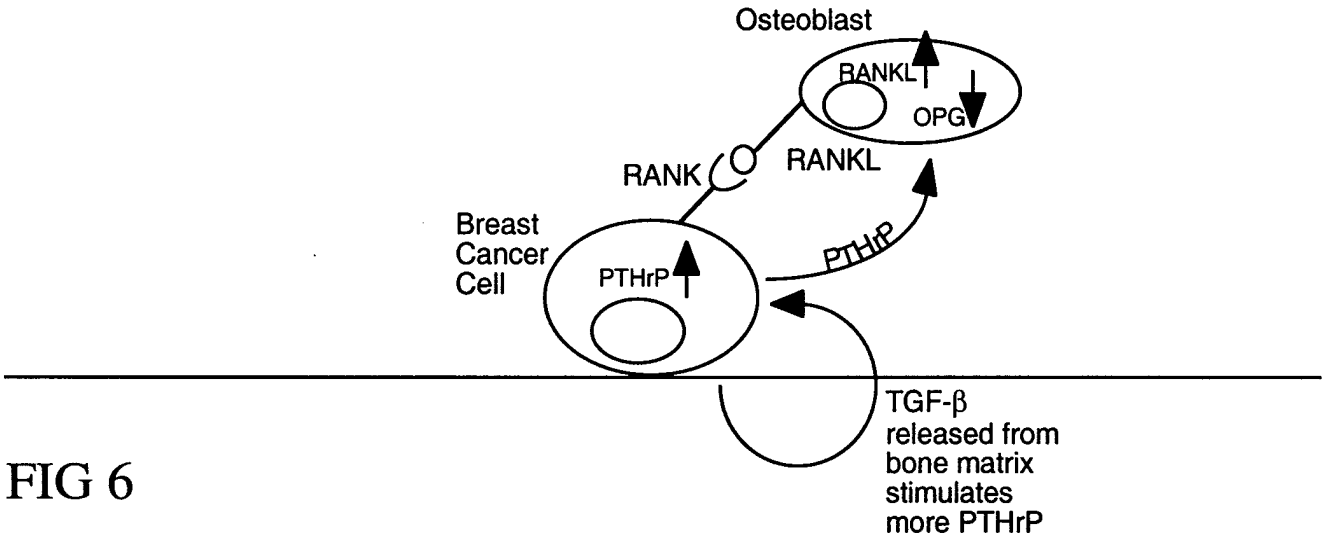
E

Figure 5. Non-neoplastic Breast Stained with Anti-RANK and Anti-RANKL Antibody

A RANK (20X)
B RANKL(20X)
C RANK(40X)
D RANKL(40X)
E Negative Control



Model 1:
Breast cancer cells secrete PTHrP that stimulate osteoblasts that in turn recruit osteoclasts to form osteolytic lesions



Model 2:
Breast cancer cells secrete PTHrP that stimulate osteoblasts that then stimulate the breast cancer cells to initiate osteolysis directly

FIG 6